Equine severe combined immunodeficiency: A defect in V(D)J recombination and DNA-dependent protein kinase activity

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ABSTRACT V(D)J rearrangement is the molecular mechanism by which an almost infinite array of specific immune receptors are generated. Defects in this process result in profound immunodeficiency as is the case in the C.B-17 SCID mouse or in RAG-1 (recombination-activating gene 1) or RAG-2 deficient mice. It has recently become clear that the V(D)J recombinase most likely consists of both lymphoid-specific factors and ubiquitously expressed components of the DNA double-strand break repair pathway. The deficit in SCID mice is in a factor that is required for both of these pathways. In this report, we show that the factor defective in the autosomal recessive severe combined immunodeficiency of Arabian foals is required for (*i*) V(D)J recombination, (*ii*) resistance to ionizing radiation, and (*iii*) DNA-dependent protein kinase activity.

During early lymphoid differentiation distinct gene segments called variable (V), diversity (D), and joining (J) are joined to form the coding sequences of immunoglobulin and T-cell antigen receptor variable regions. This process depends upon site-specific somatic recombination and results in the random assortment of various combinations of V, D, and J gene segments (reviewed in refs. 1–3). The rearrangement process involves two double-stranded DNA cuts and subsequent religations. This results in the formation of two new DNA joints—coding joints, which contain the coding information, and signal joints, which contain the two recombination signal sequences (ref. 3 and references therein). In 1989 and 1990, two highly conserved genes were discovered, RAG-1 and RAG-2 (recombination-activating genes 1 and 2), which are clearly essential for V(D)J recombinase activity (4, 5) and have recently been directly implicated in initiation of V(D)J recombination (6).

In 1983, Bosma *et al.* (7) described a spontaneous mutation in C.B-17 mice which phenotypically resembled a human lymphoid deficiency disease, severe combined immunodeficiency (SCID). In 1986, it became clear that the deficiency in C.B-17 mice is in the step of coding-joint ligation of V(D)J recombination (8–10). In 1990 it was reported that the mutation in SCID mice not only affects V(D)J recombination but also impairs the more general process of double-strand break repair (DSBR) (11, 12). Thus, this was the first suggestion that the V(D)J recombinase might utilize ubiquitous DNA repair factors to carry out the V(D)J recombination reaction.

DSBR is vital to all organisms because it ensures integrity of chromosomes. Studies with mammalian cells that are hypersensitive to agents that induce chromosomal breaks have defined three distinct complementation groups that are deficient both in rejoining double-strand breaks and in their ability to support RAG-induced V(D)J recombination (xrs, XR-1,

and murine SCID; refs. 13 and 14). Thus, the emerging consensus is that the V(D)J recombinase consists of both lymphoid-specific factors and components of the DSBR pathway. It is unclear how many distinct factors are shared between these pathways, though a minimum of three have been implicated to date. One of these has been defined as the 80-kDa subunit (p80) of the autoantigen Ku, a nuclear protein that has the interesting characteristic of binding double-stranded DNA ends in a sequence-independent manner (15–17). The Ku heterodimer interacts with an $\approx$450-kDa protein to generate a complex which is a protein-serine/threonine kinase (DNA-dependent protein kinase, DNA-PK); this kinase is dependent on DNA ends for activation (18–20). Thus, the Ku protein is the DNA-binding subunit for the $\approx$450-kDa catalytic subunit of DNA-PK (21, 22). Recently, this $\approx$450-kDa protein (DNA-PK$_{CS}$) was implicated as the molecular defect in murine SCID (23, 24). Moreover, cell lines derived from SCID mice and cell lines deficient in Ku are deficient in DNA-PK activity (23–26), and a radiation-sensitive human cell line is specifically deficient in DNA-PK$_{CS}$ (27).

SCID in Arabian foals was initially reported in 1973 (28). It is currently recognized as an autosomal recessive mutation which results in primary immunodeficiency (ref. 29 and references therein). The phenotypic characteristics of this disorder are remarkably homogeneous among affected individuals (29) and are outlined in Table 1. Most notably, these horses have severely depressed numbers of both B and T lymphocytes, whereas natural killer cell activity is normal in SCID foals (30). As is apparent from Table 1, the equine SCID phenotype is almost completely analogous to that found in mice which are homozygous for the murine SCID mutation. Thus, we assessed V(D)J recombination, radiation sensitivity, DNA end-binding activity, DNA-PK activity, and DNA-PK$_{CS}$ expression in tissues and cell lines derived from SCID foals and normal animals. We conclude that the defective factor(s) responsible for equine SCID is required for V(D)J recombination, resistance to ionizing irradiation, and DNA-PK activity and is most likely a defect in the expression of DNA-PK$_{CS}$. However, the mutation in equine SCID is phenotypically distinct from that in murine SCID in that both signal- and coding-joint ligation are impaired in SCID foals.

MATERIALS AND METHODS

Cell Lines. The 0176 and 1776 fibroblast cell lines were derived from normal horses. The 1826 and 1826P3 fibroblast

Table 1. Phenotypic characteristics of equine and murine SCID

	Equine SCID	Murine SCID
Immunologic		
B cells	Absent	Absent
T cells	Absent	Absent
NK cells	Normal activity	Normal numbers
Ig synthesis	Absent	Minimal
Pathologic		
Thymus	Severe hypoplasia	Severe hypoplasia
Peripheral lymphoid tissues	Severe hypoplasia	Severe hypoplasia
Pathogenesis	Death by $\approx$3 months	Death by $\approx$1 year
Inheritance	Autosomal recessive	Autosomal recessive

Based on information in refs. 7, 28, 29, 30 and references therein. NK, natural killer.

cell lines were derived from an Arabian foal both of whose parents were known to be heterozygous for the SCID mutation. The 1863 and 1821 fibroblast cell lines were derived from homozygous SCID foals.

Analysis of Endogenous Immunoglobulin Light-Chain Rearrangements. Spleen DNA from two different SCID foals— one unaffected Arabian foal and one unrelated horse—was isolated by utilizing DNA lysis buffer and proteinase K (Applied Biosystems). PCR conditions were 1.5 min at 94°C (denaturation), 2 min at 56°C (annealing), and 1 min at 72°C (extension) for 40 cycles (V_λ-J_λ rearrangements and germline V_κ amplification) or 50 cycles (V_κ-J_κ rearrangements). Amplified DNA was analyzed by Southern hybridization. Sequences of oligonucleotides used as primers or probes were based on published sequences (31, 32) and are as follows: 5'V_λ, 5'-CAACAGATCCCAGGAACAGCC-3'; 3'J_λ, 5'-ACCAC-CTGCGATGGTCAGGTG-3'; 5'V_κ, 5'-GGGGTCCCT-GACCGATTCAGT-3'; 3'V_κ, 5'-GAAGAATGTGCAC-ATAGATG-3'; 5'J_κ, 5'-TGTTTCGGTGATAAGTTCA-GGAGA-3'; 3'J_κ, 5'-GTGTACTCACGTTTGATCTCCAG-3'; V_λ probe, 5'-TCTCTGGCTCCAAGTCTGGC-3'; V_κ probe, 5'-ATCAGCAGCCTCCAGGCTGAAGAT-3'.

Analysis of RAG-Induced Recombination in Horse Fibroblasts. Plasmids containing the RAG-1 and RAG-2 cDNAs were generously provided by David Schatz and David Baltimore (4, 5). The RAG cDNAs were subcloned into the eukaryotic expression vector pREP9 (Invitrogen). The resulting plasmids were cotransfected with the pJH201 recombination substrate (ref. 33; gift of Joanne Hesse and Martin Gellert) via electroporation. The standard recombination assay could not be performed with these cell lines because the pJH201 plasmid does not replicate in horse fibroblasts as judged by an $\approx$2500-fold reduction in plasmid recovery as compared to mouse fibroblasts (after controlling for transfection efficiency) and an absolute lack of *Dpn* I-resistant plasmids recovered (data not shown). Thus, plasmid DNA was recovered by Hirt supernatant extraction, and recombination was assessed by PCR of Hirt fractions. PCR conditions were 1.5 min at 94°C, 2 min at 56°C, and 3 min at 72°C for 40 cycles with one primer pair. Typically, a second nested PCR was performed with 10 μl of the initial reaction mixtures. Sequences of the oligonucleotide primers are as follows: *lac* outer, 5'-ACCATGATTACGCCAAGCTTGGCTGCAG-3'; *lac* inner, 5'-TTGTTCCAGTCTGTAGCACTGTG-3'; *cat* outer, 5'-ATATCCAGTGATTTTTTTCTCCATTTTAGC-3'; *cat* inner, 5'-TTCCTTAGCTCCTGAAAATCT-3'.

Assessment of Radiation Sensitivity. Cells (2×10^3) were exposed to various amounts of ionizing radiation and immediately seeded in complete medium containing 20% fetal bovine serum. After 12 days, cell colonies were fixed and stained, and colony numbers were assessed.

DNA-PK Assays. DNA-PK assays were performed as described (25). In brief, DNA-PK activity in various cell extracts was assessed by phosphorylation of wild-type and mutant p53 peptides. In each case, relative phosphorylation was determined by comparing kinase reactions where no peptide, wild-type peptide, or mutant peptide was added.

Immunoblot Analysis of DNA-PK$_{CS}$. Equivalent amounts of extracts derived from the 0176, 1826, and 1863 cell lines were electrophoresed in an SDS/5.5% polyacrylamide gel and transferred to poly(vinylidene difluoride) membrane. Monoclonal antibodies 42psc, 25-4, and 18-2 (gifts of T. Carter; ref. 23) were used in combination as primary antibodies (1:1000 dilution of ascites), and a goat anti-mouse IgG conjugated to horseradish peroxidase (Cappel) was used as the secondary antibody. The membrane was then incubated with a chemiluminescent substrate (Renaissance; DuPont) according to the manufacturer's recommendations.

Reverse Transcription (RT)-PCR Analysis of DNA-PK$_{CS}$ RNA. RNA from 0176, 1826, and 1863 cells (2×10^6) was prepared by use of RNAzol (Biotecx Laboratories; Houston) and cDNA was prepared for RT–PCR in 25-μl reaction mixtures. Three microliters of each cDNA was used in the DNA-PK$_{CS}$ amplifications and 6 μl of each cDNA was amplified in the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) amplifications. Sequences of oligonucleotides used for DNA-PK$_{CS}$ amplification were based upon those described by Blunt *et al.* (24). Initially, the $\approx$123-nt fragment was amplified from a human cell line cDNA. This fragment was cloned and sequenced, and a third oligonucleotide was synthesized to use for hybridization. The oligonucleotides used for G3PDH amplification were purchased from Clontech.

RESULTS

To initially assess V(D)J recombination in equine SCID, rearrangements of endogenous light-chain genes in two SCID foals, one unaffected Arabian foal, and one unrelated horse were assessed by PCR using spleen DNA. Unrearranged V_κ genes were easily detected in all samples (Fig. 1A). V_λ-J_λ and V_κ-J_κ rearrangements are easily demonstrated in normal horses (horses express predominately λ light chains, >90%; refs. 31 and 32). However, no V_λ-J_λ or V_κ-J_κ rearrangements could be detected in the spleen DNA from either SCID foal. Furthermore, no signal joints were detected in the DNA derived from either SCID foal, though V_κ-J_κ signal joints were easily detected in the DNA derived from normal animals. Finally, though limited sequence information is available from equine immunoglobulin heavy chains, similar results were observed in an analysis of V_H-J_H recombination in SCID and normal foals (data not shown). Impairment of signal-joint formation distinguishes equine SCID from the SCID defect of C.B-17 mice, which have deficient coding-joint ligation but relatively normal signal-joint ligation (8).

To corroborate our conclusion that V(D)J rearrangement is impaired in SCID foals and to address whether the RAG genes are defective in SCID horses, fibroblast cell lines derived from affected individuals were tested for their capacity to support V(D)J recombination after transfection with RAG-1 and RAG-2 expression vectors (Fig. 2). The pJH201 recombination substrate (which retains signal joints after rearrangement; ref. 33) was transiently transfected with or without RAG expression vectors into fibroblast cell lines derived from a normal unrelated horse, 0176; a phenotypically normal Arabian foal, 1826; and a SCID foal, 1863. These experiments were complicated because the transfected substrates do not replicate in horse cells, and the standard recombination assay requires replication of the substrate for efficient recovery of recombined substrate (*Materials and Methods*). Thus, rearrangement of the substrate was assessed by PCR analysis. A representative transfection experiment is shown. Though un-

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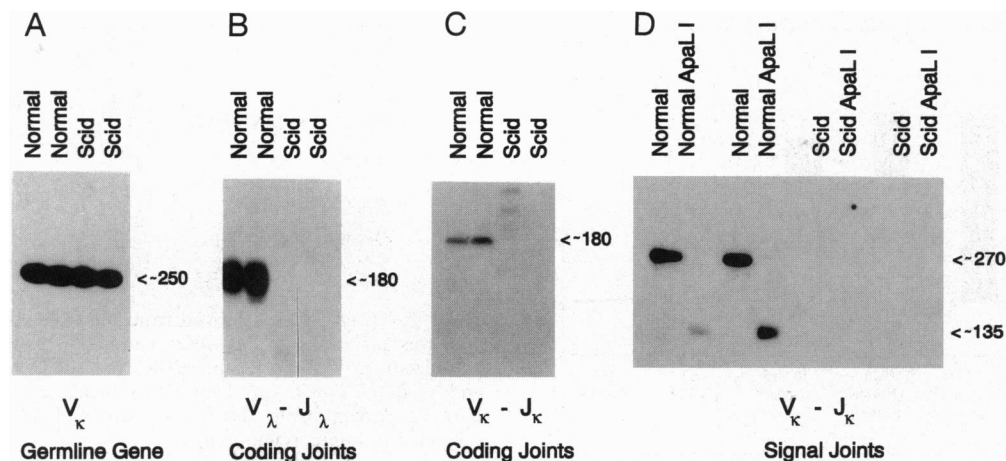*Proc. Natl. Acad. Sci. USA* 92 (1995)   11487

FIG. 1.   PCR analysis of light-chain gene rearrangement in spleen DNA derived from two different SCID foals and two phenotypically normal horses. Amplification primers were based upon equine light-chain sequences reported previously (31, 32). In each situation, 5 μg of spleen DNA was utilized. PCR products were separated in 1.8% agarose gels, transferred to nylon membranes, and hybridized to ^{32}P-labeled internal oligonucleotide probes. Unrearranged V_κ genes and V_λ–J_λ rearrangements were detectable after 40 cycles. V_κ–J_κ rearrangements required 50 cycles (horses overwhelmingly express λ light chains). (*A*) Unrearranged V_κ genes were amplified with primers within V_κ and 3' of V_κ. (*B*) V_λ–J_λ rearrangements were amplified by primers within V_λ and J_λ. (*C*) V_κ–J_κ coding joints were amplified with primers within V_κ and 3' of J_κ. (*D*) V_κ–J_κ signal joints were amplified with primers 3' of V_κ and 5' of J_κ. A portion of each amplification mixture was digested with the restriction endonuclease ApaLI as indicated to confirm authenticity of the signal joints.

rearranged pJH201 (340-bp fragments) was equivalently detectable in all three cell lines with or without RAG cotransfection, rearrangements (140-bp ApaLI-sensitive fragments) were apparent only in 0176 and 1826 cell lines that were cotransfected with RAG-1 and RAG-2 expression vectors. In one of six experiments, a very low level of rearrangement of the pJH201 substrate was detected in the 1863 cell line. These data are in agreement with the marked deficiency of rearrangement of immunoglobulin genes in SCID foals and corroborate the conclusion that the defect in SCID foals is a severe diminution in the capacity to carry out V(D)J recombination. Since V(D)J recombination remains impaired in SCID fibroblasts complemented with normal murine RAG-1 and -2, mutations in RAG-1 or RAG-2 cannot be responsible for the equine SCID defect.

Next we assessed the relative sensitivity of each of these cell lines to ionizing radiation. The 1863 and 1821 cell lines were found to be significantly more radiation sensitive than cell lines derived from phenotypically normal horses (Fig. 3). We also assessed the relative sensitivity of these cell lines to bleomycin sulfate (a DNA-damaging agent) and found that the equine SCID cell lines were also hypersensitive to this agent (data not shown).
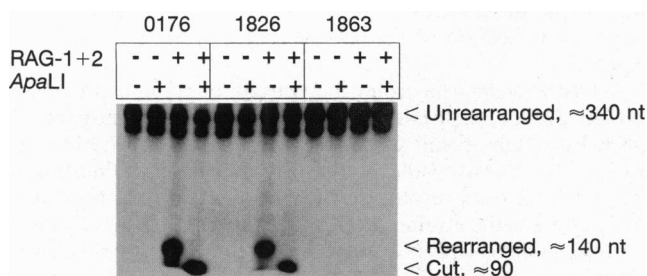

FIG. 2.   Assessment of pJH201 rearrangement in fibroblast cell lines 0176 (derived from a normal horse), 1826 (derived from an unaffected Arabian horse), and 1863 (derived from a homozygous SCID foal). Hirt-fractionated DNA was recovered from substrate (pJH201)-transfected cells (with or without cotransfection of RAG expression vectors). Unrearranged substrate generates a 340-nt amplification product; rearranged plasmids generate a 140-nt rearrangement product.

The Ku heterodimer binds free DNA ends (with either 5' or 3' protruding ends or blunt ends) in a sequence-independent manner (16, 34). The molecular defect in the Chinese hamster ovary (CHO) DSBR mutant cell line xrs6 is in end-binding activity (15, 35, 36) and in Ku p80 (17, 37). Thus, we assessed DNA end-binding activity in the fibroblast cell lines by electrophoretic mobility-shift assay and found no differences in Ku activity in equine SCID cell lines compared with normal equine fibroblasts (data not shown). To further investigate the Ku/DNA-PK$_{CS}$ complex in equine SCID cell lines, a series of DNA-PK kinase assays were performed (Fig. 4). Levels of DNA-PK activity in four normal horse cell lines (0176, 1776, 1826, and 1826P3) were similar. However, DNA-PK activity was not detectable in equine SCID cell lines (1863 and 1821),
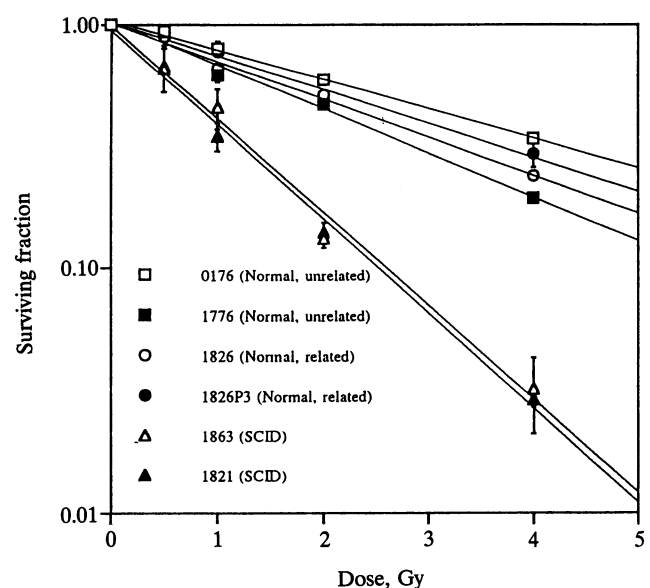

FIG. 3.   Cells (2 $\times$ 10^3) of cell lines 0176 and 1776 ($\square$ and $\blacksquare$), 1826 and 1826P3 ($\bigcirc$ and $\bullet$), and 1863 and 1821 ($\triangle$ and $\blacktriangle$) were exposed to increasing levels of ionizing radiation and then plated into 150-mm-diameter tissue culture plates. Twelve days later, cells were trypsinized and counted.
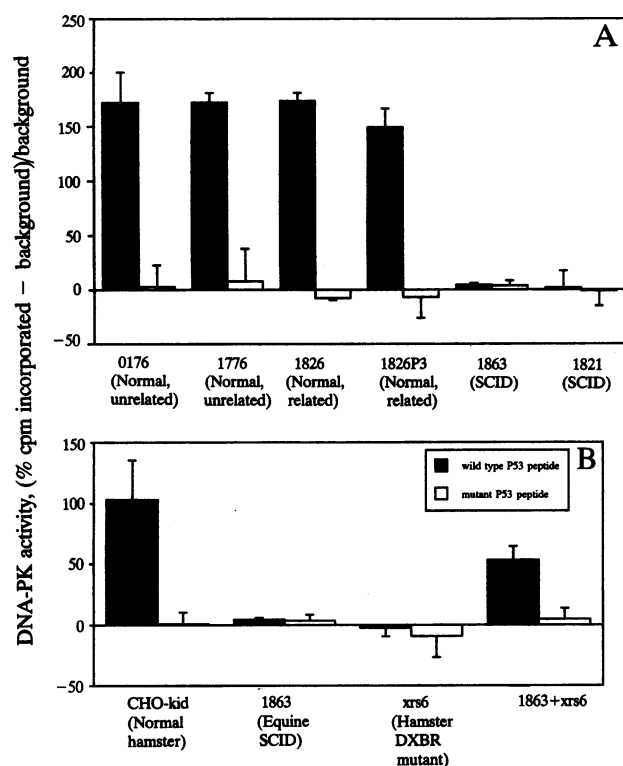
FIG. 4. DNA-PK assays as described by Finnie et al. (25). DNA-PK activity in various cell extracts was assessed by phosphorylation of wild-type (solid bars) and mutant (open bars) p53 peptides. In each case, relative phosphorylation was determined by comparing kinase reactions where no peptide, wild-type peptide, or mutant peptide was added. Relative phosphorylation was determined as percent increase over no peptide as described by Blunt et al. (24). Standard deviations are based on a minimum of three separate experiments. (A) Extracts from 0176 and 1776, fibroblasts derived from two normal horses; 1863 and 1821, fibroblasts derived from two SCID foals; 1826 and 1826P3, fibroblasts derived from a normal horse with known heterozygote parents. (B) Extracts from normal CHO cells; hamster DSBR mutant xrs6; 1863 equine SCID cells; or a mixture of xrs6 and 1863. By a paired t test and comparison of phosphorylation of wild-type and mutant peptide in the xrs6 and 1863 in vitro complementation, P = 0.0232.

suggesting that the equine SCID factor is required to establish normal levels of DNA-PK activity.

Since the phenotype of rearrangements found in equine SCID closely resembles those found in the xrs6 DSBR mutant cell line (13, 14), we performed an in vitro complementation experiment (Fig. 4B). As expected, DNA-PK activity was not detectable from the xrs6 mutant cell line as compared with activity detected from normal CHO cells. However, when extracts from the equine SCID cell line 1863 were mixed with xrs6 extracts, DNA-PK activity was detected. Thus, the defect in equine SCID cannot be in Ku p80.

Next, we assessed DNA-PK$_{CS}$ mRNA levels by RT–PCR. Levels of DNA-PK$_{CS}$ mRNA were not diminished in the SCID cell fibroblasts compared with normal fibroblasts (as controlled by G4PDH expression) (Fig. 5 Left). However, when levels of DNA-PK$_{CS}$ protein were ascertained by use of antibodies to human DNA-PK$_{CS}$ in immunoblot analyses, SCID fibroblasts were found to be defective in DNA-PK$_{CS}$ expression (Fig. 5 Right). The ≈450-kDa DNA-PK$_{CS}$ polypeptide was readily detected from normal equine fibroblasts but not from SCID fibroblasts. With long exposures very small amounts of a protein with the same electrophoretic mobility were detected in equine SCID fibroblasts. Thus, we conclude that the level of DNA-PK$_{CS}$ is significantly diminished in equine SCID cells.
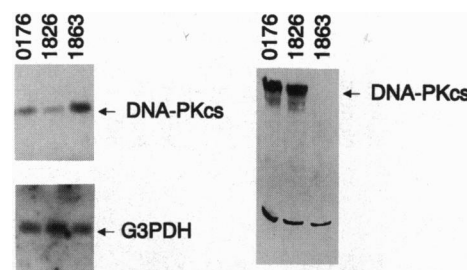

FIG. 5. (Left) cDNA derived from the 0176, 1826, and 1863 cell lines was analyzed for DNA-PK$_{CS}$ and G3PDH mRNA by Southern hybridization of RT–PCR-amplified DNA. The DNA-PK$_{CS}$ (≈123 bp) and G3PDH (≈850 bp) hybridizing bands are indicated. (Right) Extracts derived from the 0176, 1826, and 1863 cell lines were subjected to immunoblot analysis.

DISCUSSION

These data demonstrate that, as is the case with murine SCID, the mutation in equine SCID affects both V(D)J recombination and DNA-PK activity. However, the impairment of V(D)J recombination in these two immunodeficiencies differs. First, in murine SCID, though coding-joint formation is severely diminished, signal-joint ligation is normal. In contrast, in equine SCID, both signal- and coding-joint ligation are severely impaired. Furthermore, deficient signal-joint formation in SCID foals is apparent for rearrangements both of endogenous genes and of the pJH201 substrate in RAG-complemented fibroblasts derived from these animals. Second, mice homozygous for the SCID mutation are not completely incapable of carrying out coding-joint ligation, in that varying levels of relatively normal coding joints can be demonstrated in these animals ("leaky" SCID phenotype; refs. 39–41). These joints often have excessive deletions from the coding ends and also often have long P segments, consistent with the fact that broken coding ends with hairpinned termini accumulate in thymocytes from SCID mice (42–45). V$_\kappa$–J$_\kappa$ rearrangements are easily detected from spleen DNA derived from SCID mice, whereas coding-joint ligation appears to be more completely impaired in SCID foals.

Though equine SCID and murine SCID have definite phenotypic differences, the data presented here most strongly support the conclusion that DNA-PK$_{CS}$ is defective in both. Still, the phenotypic differences suggest that if DNA-PK$_{CS}$ is defective in equine SCID, the mutation differs from the mutation in murine SCID. We have directly compared the relative DNA-PK$_{CS}$ levels in normal and SCID equine cell lines as well as normal and SCID mouse cell lines and found that DNA-PK$_{CS}$ diminution was more profound in equine SCID cells than murine SCID cells (data not shown). Thus, the phenotypic differences between SCID mice and SCID foals may be a reflection of the relative depression in DNA-PK$_{CS}$ expression.

DNA-PK clearly has roles in a variety of cellular processes (18–20, 22, 23, 38, 46); distinct mutations in different parts of DNA-PK$_{CS}$ may result in different deficiencies. Another attractive hypothesis would be that the murine SCID mutation specifically affects resolution of hairpinned or damaged termini, whereas the equine SCID mutation might affect ligation of broken ends—two processes already linked to the DNA-PK complex.

Of course, it is possible that the equine SCID defect is not in DNA-PK$_{CS}$. The finding that DNA-PK$_{CS}$ mRNA levels are not diminished in equine SCID cells, but protein levels are, suggests that DNA-PK$_{CS}$ in SCID horses is unstable. An alternative explanation for our results is that the mutation in equine SCID does not cause a functional change in DNA-PK$_{CS}$ but, rather, alters the stability of DNA-PK$_{CS}$. It has recently been reported that the relative stability of Ku p80 is dependent

upon the presence of Ku p70 (47). It is possible that DNA-PK$_{CS}$ stability requires another factor which could be an alternative candidate for the defect in equine SCID.

In summary, we have demonstrated that the autosomal recessive defect that results in SCID in Arabian foals is explained by defective V(D)J recombination. Furthermore, cells derived from SCID horses cannot support RAG-induced V(D)J recombination, are hypersensitive to ionizing radiation, lack detectable DNA-PK activity, and have diminished levels of DNA-PK$_{CS}$.

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